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Location of the calcium binding site in Photosystem II: a Mn^{2+} substitution study

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Abstract

The whereabouts of the Ca^{2+} site in Photosystem II (PSII) was investigated by experiments in which Mn^{2+} was substituted for Ca^{2+}. When stoichiometric amounts of Mn^{2+} ions were added to Ca^{2+}-depleted PSII, the Mn^{2+} was not detected by EPR. The titration of Ca^{2+} back into Ca^{2+}-depleted/Mn^{2+}-containing PSII resulted in the simultaneous release of the Mn^{2+} and the loss of the two EPR signals which are characteristic of the Ca^{2+}-depleted enzyme (i.e., the stable, modified S_2 multiline signal arising from the intrinsic Mn cluster and the split S_3 signal from an organic radical interacting with the Mn cluster). These results indicate that the Mn^{2+} occupies the functional Ca^{2+} site. The S_2 and S_3 EPR signal characteristic of this kind of Ca^{2+}-depleted preparation were unaffected by the binding of the Mn^{2+}. Since, from earlier results, it seems likely that the modification and stability of S_2 multiline signal in these PSII preparations is due to binding of chelator to or close to the Mn cluster, the present results indicate that the Ca^{2+} site (at least when occupied by Mn^{2+}) does not overlap with the chelator binding site. Since Mn^{2+} binding does not effect the S_2 EPR signal from the Mn cluster, it can be concluded that the Mn^{2+} is not involved in detectable magnetic interactions with the cluster. This result indicates that the Mn^{2+}-occupied Ca^{2+} binding site is outside the first co-ordination sphere of the Mn cluster. The relaxation properties of TyrD$^{\cdot}$ were enhanced by the presence of the Mn^{2+} when the Mn cluster was in the S_1 state.

Keywords: Photosystem II; Calcium; Binding site; Oxygen evolution

1. Introduction

The role of Ca^{2+} in the O_2-evolving enzyme has been the focus of much research in recent years (reviews: Refs. [1–4]). In the absence of Ca^{2+}, electron transfer from TyrZ to the photo-oxidised chlorophyll, $P680^+$, is slowed down [5] (see also Ref. [6]) and an abnormal S_3 state, an organic free radical, is formed [7] (see also Ref. [8]). After formation of this S_3 state, further electron donation to $P680^+$ seems to be unable to compete with back-reactions and so the charge accumulation cycle is blocked [5].

There has been some debate over the number of intrinsic Ca^{2+} sites associated with O_2 evolution [1–4]. A degree of consensus has been established that one Ca^{2+} site is associated with the Ca^{2+} deple-

Abbreviations: PSII, Photosystem II; PPBQ, phenyl p-benzoquinone; EGTA, ethylene glycol bis (β aminoethyl ether)-*N,N,N′,N′*, tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tyr, tyrosine.

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tion effects generally reported [9–12]. A second Ca^{2+} site seems to be associated with a light-harvesting protein and it is not clear if this site is related to oxygen evolution [9,13], although it does seem clear that the usual procedures used to remove and reconstitute the oxygen evolution-dependent Ca^{2+} do not involve the Ca^{2+} which binds to the light-harvesting protein [9].

Although the role of Ca^{2+} is unclear, it has been suggested that it may play a role in regulating the electrostatic constraints involved in charge accumulation (e.g., the modulation of deprotonation and ion binding) [5]. This suggestion was based on observations of differences in the Ca^{2+} binding properties depending on the charge accumulation cycle [14] and on indications that proton release was inhibited by Ca^{2+}-depletion [15]. Additional or alternative roles for Ca^{2+} in controlling substrate and Cl^- access to the active site have been prompted by a range of enzymological and spectroscopic studies [16] (see also Refs. [1–4,17,18]).

An improved understanding of the chemical role of Ca^{2+} requires knowledge of the actual location of its binding site on the enzyme. Metal substitution experiments have already contributed to this area. It was shown that Sr^{2+} can reconstitute 40% of the activity of Ca^{2+} [19] and this was subsequently shown to be due to a slow down in the rate-limiting step of O_2 evolution [20] which was also manifest by a slow-down in oxidation of the S-states [5]. Sr^{2+} substitution also resulted in a major modification of the EPR spectrum of the Mn cluster, prompting the suggestion that Ca^{2+} may be physically close to the Mn cluster [20]. EXAFS experiments comparing the Ca^{2+} and Sr^{2+}-containing enzyme [21] showed a feature at 3.3 Å which was modified in the Sr^{2+}-substituted material, thus leading to the conclusion that the back-scatterer at 3.3 Å was probably Ca^{2+} [21]. This conclusion has been disputed in a subsequent EXAFS study in which the back-scatterer at 3.3 Å was unaffected by Sr^{2+} replacement [22]. The origin of this discrepancy is not yet clear.

Studies of PSII photoactivation [23–26], the process by which the Mn cluster is assembled, showed that both Ca^{2+} and Mn^{2+} ions are required for enzyme activity and that these ions compete for their respective binding site(s) [23,25,26]. When photoactivation was done without Ca^{2+}, a non-functional enzyme containing 5–6 Mn^{2+} ions was obtained which, upon subsequent addition of Ca^{2+}, became activated with the loss of 1–2 Mn^{2+} ions [26]. It seemed clear then that under photoactivation conditions the Mn^{2+} ion could occupy the Ca^{2+} site.

In the present work, we attempted to exchange Mn^{2+} for Ca^{2+} using Ca^{2+}-depletion procedures. A method is reported by which this exchange seems to have been achieved. Evidence is reported indicating that one Mn^{2+} can be specifically bound in the Ca^{2+} site. The properties of the Mn^{2+}-substituted enzyme were then studied in order to obtain information on the whereabouts of the Ca^{2+} site.

2. Materials and methods

PSII membranes were prepared from spinach chloroplasts essentially as described in Ref. [27] and these were depleted of Ca^{2+}, resulting in essentially complete inhibition of oxygen evolution as described in Ref. [7]. In this preparation, the 17- and 23-kDa are reconstituted after Ca^{2+} depletion. The resulting Ca^{2+}-depleted membranes were resuspended in 0.3 M sucrose, 25 mM MES (pH 6.5) at chlorophyll concentrations of 6–10 $mg \cdot ml^{-1}$. Ca^{2+}-depleted membranes were dark-adapted for 1 h prior to addition of Mn^{2+}, and unless otherwise stated, they were then incubated with 1 Mn^{2+} per PSII reaction centre (on the basis of 200 chlorophylls per PSII reaction centre), for 2 h in the dark on ice. 1 mM phenyl-p-benzoquinone (PPBQ) (dissolved in dimethyl sulphoxide) was added after Mn^{2+} addition, to act as an electron acceptor in illumination experiments to form the S_3 state. For Fig. 2, a known concentration of Ca^{2+}, between 200 and 2700 Ca^{2+} per PSII reaction centre (approx. 5–75 mM Ca^{2+}), was then added and the sample left for 30 min. on ice in the dark. Separate samples were used for each Ca^{2+} concentration. All additions to the Ca^{2+}-depleted membranes were carried out in total darkness. Mn^{2+} and Ca^{2+} were added from stock solutions of their chlorides.

In the experiments which required the formation of S_1 in Ca^{2+}-depleted, chelator-treated PSII membranes, samples were incubated in the dark at 0°C for three days to allow the stable S_2 state to decay [28]. This was done using samples which contained one

added Mn^{2+} per centre and as a control, using samples with no added Mn^{2+}. EPR signals attributable to the Mn^{2+} were not detected by c.w. EPR under the conditions used. The long incubation resulted in the loss of the stable TyrD$^{\cdot}$ signal from approx. 40% of the centres. The TyrD$^{\cdot}$ and the stable S_2 multiline signal could be regenerated to close to 100% in such samples upon illumination at 0°C followed by a period of dark adaptation (not shown).

The S_3 state was formed by illumination of PSII membrane samples for 2 min in an ethanol bath cooled to 0°C with solid CO_2 [7]. EPR spectra were recorded with a Bruker ER 200 spectrometer equipped with an Oxford Instruments cryostat. The Mn^{2+} EPR signal was quantified by comparison of the amplitude of the six line Mn^{2+} EPR signal at 30 K, with that from an acid-denatured PSII membrane sample. After acid treatment the Mn ions originating in the cluster were detected in the medium as Mn^{2+} and were equivalent to 4 Mn^{2+} per PSII reaction centre as determined by atomic absorption (Perkin Elmer) on comparable samples.

In some control experiments active PSII membranes were washed in buffer containing 0.4 M sucrose, 10 mM NaCl, 25 mM MES (pH 6.5) and 10 mM EGTA. This treatment does not affect activity but is done in order to deplete divalent cations from other potential binding sites. One Mn^{2+} per reaction centre was added to this kind of preparation under conditions as described above.

3. Results

EPR studies of Ca^{2+}-depleted, chelator-treated PSII membranes to which Mn^{2+} in stoichiometric amounts had been added, showed that these membranes exhibit a dark, stable S_2 multiline signal (Fig. 1). Illumination of the membranes at 0°C, which results in formation of the S_3 state, gave the EPR signal shown in the inset of Fig. 1. The S_2 and S_3 EPR signals shown in Fig. 1 are characteristic of Ca^{2+}-depleted, chelator-treated, PSII membranes which contain no exogenous Mn^{2+} [7]. Thawing and incubating the Mn^{2+}-containing membranes in the dark on ice for 30 min caused the loss of the S_3 signal and reappearance of the S_2 multiline signal like that shown in Fig. 1 (see Ref. [7]). Under the conditions

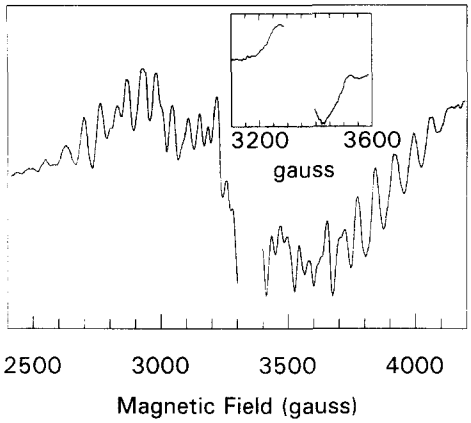

Fig. 1. Dark-stable S_2 multiline EPR signal from Ca^{2+}-depleted/chelator treated PSII membranes in the presence of one added Mn^{2+} per PSII reaction centre and PPBQ (1 mM) as an electron acceptor. The sample incubated in darkness for 3 h prior to freezing as described in Section 2. Instrument settings: microwave power 31 mW, modulation amplitude 20 G, temperature 10 K. Inset: S_3 EPR signal from a similar sample (Ca^{2+}-depleted/chelator-treated PSII membranes containing one added Mn^{2+} per PSII reaction centre and 1 mM PPBQ) following illumination at 0°C for 2 min. Instrument settings: microwave power 2 mW, modulation amplitude 3 G, temperature 10 K.

used, no new EPR signals could be observed from the exogenous Mn^{2+} (at temperatures between 4 K and 30 K). Identical results were found whether the Ca^{2+}-depleted membranes were incubated with exogenous Mn^{2+} for 5 min, 1 h or 2 h, as well as for a control sample containing no added Mn^{2+}. No broadening of the hyperfine lines of the S_2 EPR signal nor of the S_3 EPR signal could be detected under the conditions used. We conclude from these results that addition of Mn^{2+} to Ca^{2+}-depleted membranes has no apparent effect on the S_2 and S_3 states as monitored by EPR spectroscopy.

The Mn^{2+} which had been added to Ca^{2+}-depleted PSII membranes was released from the membranes when they were reconstituted with Ca^{2+}. It was found that the stable S_2 multiline signal disappeared after incubation of the Mn^{2+}-containing, Ca^{2+}-depleted samples with 10 mM Ca^{2+} (equivalent to approx. 300 Ca^{2+} per reaction centre) for 2 h in the dark on ice, and an EPR signal consisting of six lines (average spacing approximately 90 G) was observed. This signal is characteristic of Mn^{2+} in aqueous solution and was equivalent to 1 ± 0.2 Mn^{2+} per PSII reaction centre. Illumination of the sample at 200 K

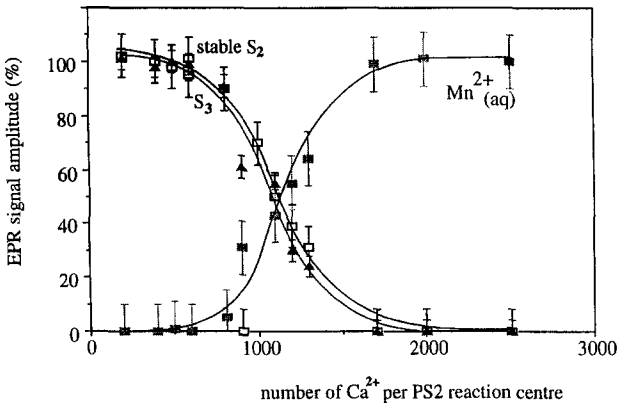

Fig. 2. Effect of addition of Ca^{2+} to Ca^{2+}-depleted PSII membranes containing one added Mn^{2+} per PSII reaction centre. After addition of Ca^{2+}, the sample was incubated for 30 min in darkness on ice prior to freezing. Open squares: amplitude of stable S_2 multiline EPR signal, filled triangles: amplitude of S_3 EPR signal, filled squares: amplitude of the six line EPR signal attributed to Mn^{2+} in solution. Each point is the average 3 measurements and the error bars shown the standard deviation. S_2 and S_3 signals measured as in Fig. 1, Mn^{2+} was measured using the following instrument settings: microwave power 20 μW, microwave amplitude 20 G, temperature 30 K. 1000 Ca^{2+} per reaction centre corresponds to approx. 27 mM Ca^{2+}.

resulted in an S_2 multiline signal typical of untreated, (Ca^{2+}-containing) PSII membranes. The amplitude of this S_2 multiline signal was about 80–90% of that observed for untreated PSII membranes (not shown).

To determine if the Ca^{2+}-induced release of Mn^{2+} into aqueous solution correlated with reconstitution of activity in PSII, Ca^{2+} was titrated back into Mn^{2+}-containing, Ca^{2+}-depleted membranes. Fig. 2 shows the decrease in the amplitudes of both the stable S_2 and S_3 EPR signals as increasing concentrations of Ca^{2+} were added to Mn^{2+}-containing, Ca^{2+}-depleted membranes. The loss of the ability to trap the S_3 signal after illumination at 0°C shows that the enzyme cycle is no longer blocked after formation of S_3. Also shown is the simultaneous increase in the Mn^{2+} EPR signal. The maximum size of the Mn^{2+} signal was measured in each sample after the addition of excess Ca^{2+} (up to 100 mM) and it corresponded to the release of between 0.9–1.2 Mn^{2+} per PSII reaction centre. In the titration, the appearance of Mn^{2+} in solution correlates with the loss of the EPR features which are characteristic of Ca^{2+}-depletion. This indicates that the Mn^{2+} is bound in the Ca^{2+} site prior to Ca^{2+} addition.

In the titration experiment the concentrations of Ca^{2+} required were higher than expected based on preliminary time course studies (not shown but see Ref. [7] for time course of decay of stable multiline upon addition of Ca^{2+}) and based on earlier results like those described above in which 300 Ca^{2+} per PSII incubated for 2 h was sufficient for approximately 100% decay of the stable S_2 and Mn^{2+} release. The main difference between the titrations and the other experiments is that in the titrations the samples underwent a single freezing step (separate samples were used for each concentration) while the other experiments involved additional freeze-thaw cycles for the measurement of the stable multiline prior to Ca^{2+} addition and during the incubation time course. It seems possible that freeze-thaw cycles could accelerate Ca^{2+} binding perhaps due to perturbation of the extrinsic proteins.

As a control experiment for the possibility that the Mn^{2+} binding may be some kind of non-specific effect, one Mn^{2+} per PSII was added to PSII membranes which had been washed in chelator (10 mM

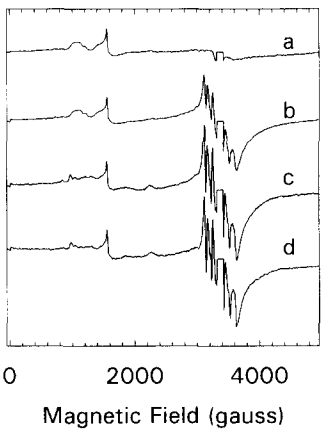

Fig. 3. The effect of the addition of 1 Mn^{2+} to functional (not Ca^{2+}-depleted) PSII which had been pre-washed with EGTA (10 mM). EGTA was removed from the sample by a series of washings (i.e., pelleting the membranes followed by resuspending in EGTA-free buffer). (A) Intact PSII after washing with EGTA. (B) The same sample as A but after addition of 1 Mn^{2+} per PSII and incubation in darkness for 2 h at 0°C. (C) As per B but a different sample (intact PSII after the addition of 1 Mn^{2+} per PSII followed by incubation for 2 h in darkness at 0°C). (D) Same sample as C but with after the addition of 50 mM Ca^{2+} and incubation for 30 min in darkness at 0°C. Instrument settings: microwave power, 1 mW, modulation amplitude 24 gauss, temperature 15 K.

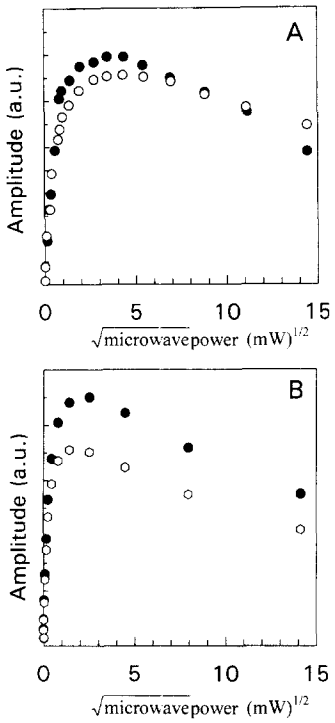

Fig. 4. Effect of the presence of 1 Mn^{2+} per PSII added to Ca^{2+}-depleted/chelator-treated PSII on the microwave saturation properties of TyrD·. Panel A shows saturation plots of TyrD· in the presence of the dark-stable S_2 manganese multiline signal with (solid circles) and without (open circles) 1 added Mn^{2+} per centre. Panel B shows similar plots but after dark incubation for 3 days in order to form the S_1 state. Instrument settings were as follows: modulation frequency 12.5 KHz, temperature 15 K.

EGTA) under conditions in which the intrinsic Ca^{2+} was not removed (i.e., without salt-washing). Under these conditions the Mn^{2+} added was detectable as a typical six-line Mn^{2+} EPR signal (Fig. 3b). As a further control Ca^{2+} (50 mM) was added to a Mn^{2+}-containing EGTA-washed PSII sample. No additional Mn^{2+} became detectable under these conditions (Fig. 3d). This is a further indication that when Mn^{2+} is added to Ca^{2+}-depleted PSII, it occupies a specific Ca^{2+} site.

The microwave saturation properties of the TyrD· signal are dominated by the relaxation of the Mn cluster under most conditions [29,30]. Fig. 4A shows that in the S_2 state, the presence of the exogenously added Mn^{2+} has little effect on saturation properties of TyrD·. Since the S_2 state is present in both samples and this is the dominant relaxer, an effect of an extra relaxer in the form of Mn^{2+} may be expected to be difficult to detect. Therefore, the relaxation study was repeated in the presence of the dark-adapted S_1 state which is known to be the slowest relaxer of the S states [30]. The samples were allowed to relax back to the S_1 state by incubation for 3 days at 0°C in the dark and the relaxation properties of TyrD· were studied. It was found that the sample containing Mn^{2+} showed a marked relaxation enhancement compared to the control sample (Fig. 4). It seems likely that this effect is due to a dipolar coupling between TyrD· and the Mn^{2+} in the Ca^{2+} site. The relaxation enhancement on the TyrD· from the Mn^{2+} is smaller than that from that arising from the Mn cluster in the S_2 state. When Mn^{2+} was added to EGTA-washed but otherwise untreated PSII, a slight relaxation enhancement is observed in the S1 state (not shown) but the effect is much less marked than the effect seen in Fig. 4B.

4. Discussion

The results indicate that Mn^{2+}, when added in stoichiometric amounts to Ca^{2+}-depleted PSII membranes, binds to a site that renders it undetectable by conventional EPR (although preliminary data indicate that it is detectable by pulsed EPR [unpublished]). It is well known that Mn^{2+} can become virtually undetectable by conventional EPR when it has an asymmetric ligand environment. This bound Mn^{2+} is displaced by the addition of Ca^{2+}. By titrating the amount of Ca^{2+} added back to the PSII membranes, a correlation was seen between the appearance of Mn^{2+} in solution, the loss of both the stable S_2 multiline EPR signal and the loss of the ability to form the EPR signal characteristic of S_3 in the inhibited enzyme. This correlation is taken as an indication that the Mn^{2+} occupies the functional Ca^{2+} site.

It is possible that the Mn^{2+} may be bound in a site other than that normally occupied by the Ca^{2+} and that Mn^{2+} may be displaced from this site by Ca^{2+} at a concentration which is coincidentally identical to that required for Ca^{2+} to occupy its site that is related to O_2 evolution. Apart from this being a somewhat unlikely coincidence, the control experiment, in which we tested for Mn^{2+} binding to EGTA-washed PSII that had not been depleted of the activity-related

Ca^{2+}, showed no evidence of such a Mn^{2+} binding site. An alternative possibility is that the binding of Ca^{2+} to its site results in release of Mn^{2+} from a different site, i.e., an allosteric effect. Although these possibilities cannot be ruled out, the most straightforward interpretation of the results is that Mn^{2+} occupies the Ca^{2+} site that is associated with O_2 evolution.

From photo-activation studies, it is known that Mn^{2+} has a much greater affinity for the Ca-site than does Ca^{2+} itself [25,31]. This seems consistent with the observation that the addition of stoichiometric quantities of Mn^{2+} to Ca^{2+} depleted PSII can lead to the specific occupation of the Ca site.

The concentration of Ca^{2+} required to displace Mn^{2+} from the site and to reactivate the enzyme was found to be high, approx. 32 mM. Similar concentrations of Ca^{2+} were used to displace the extra Mn^{2+} when photo-activation was done without Ca^{2+} [26]. These concentrations are higher than might be expected based on the known binding constants for Ca^{2+} reactivation of O_2 evolution (50–100 μM and 1–2 mM [32,33]; n.b., smaller binding constants have been reported in the absence of the cations which may compete for the site, see Refs. [2,12]). However, these values are not necessarily relevant under the conditions of the experiments here and in ref. [26] since it is known that Ca^{2+} binding is dependent on the S-states and this dependence seems to reflect changes in the accessibility of the Ca^{2+} site as well as potential S-state dependencies in the binding constant [14]. In the present experiments and those in ref. [26], Ca^{2+} was added in darkness, i.e., in the S_1 state, and under such conditions Ca^{2+} binding is expected to be slow [14]. In addition, the PSII membranes used here had been reconstituted with the extrinsic polypeptides and these seem to play an important role in limiting access to the Ca site [1,2].

Since the S_2 Mn multiline EPR signal and the S_3 signal are not noticeably affected by the presence of the bound Mn^{2+}, then we conclude that the added Mn^{2+} is not involved in a significant magnetic interaction with the intrinsic Mn cluster. This result seems then to argue against models in which the Ca^{2+} (and hence the added Mn^{2+}) shares the same co-ordination sphere as the Mn cluster (e.g., Refs. [2,16,21].

The indications in the literature that the Ca^{2+} site is close to the Mn site are the following:

(1) Ca^{2+} removal and reconstitution have very marked effects on the functioning of the O_2 evolving enzyme [1–4].

(2) Replacement of Ca^{2+} by Sr^{2+} results in marked changes in the Mn EPR signals [20] which are very reminiscent of the modifications caused by the binding of NH_3 to the Mn cluster [34] (see Ref. [35]).

(3) Removal of Ca^{2+} allows further modification of the Mn cluster, thought to be due to binding of chelators [36–38], resulting in stabilisation of the S_2 state as well as major modification of its spectral properties [7].

(4) The Ca^{2+} binding properties are modulated by the S-states (i.e., the redox state of the Mn cluster) [14].

(5) EXAFS results of Latimer et al. [21] indicate that Ca^{2+} is located 3.7 Å from the Mn cluster (but see Ref. [22]).

(6) Extrinsic electron donors can more easily attack the Mn cluster when Ca^{2+} is absent [39,40].

With the exception of point 5 (which is disputed in ref. [22]), all the other indications are indirect and do not require Ca^{2+} to be within the co-ordination sphere of the Mn cluster. The present results seems to indicate that the Ca^{2+} site is outside the co-ordination sphere of the Mn cluster (at least when it is not occupied by the Ca^{2+}), but this would be quite consistent with the Ca^{2+} site only being a short distance outside the first co-ordination sphere of the cluster. It should be pointed out, however, that given that protein conformational changes are so often associated with Ca^{2+} binding, it is also quite possible that the observations showing an association between Ca^{2+} effects and the Mn cluster can be explained by interactions occurring through the protein over a longer distance.

On the location of the Ca^{2+} site, the present results only allows us to suggest where it is not: i.e., very close to the Mn cluster. Even that negative conclusion must be qualified. Since we consider it possible that Ca^{2+} binding may result in a conformational change in the PSII proteins, we should also consider the possibility that such a change could directly influence the distance between the Ca^{2+} and the Mn cluster. It is even possible then that, although the Mn^{2+}-occupied Ca^{2+} site may be outside the co-ordination sphere of the Mn cluster in the inhibited enzyme, binding of Ca^{2+} could bring the Ca^{2+}

site closer to the Mn cluster, resulting in a shared co-ordination in the native enzyme.

We have argued previously that the modification of the S_2 multiline signal and its unusual stability are not simply due to an effect of Ca^{2+}-depletion but, instead, result from binding of chelators directly, or close to the Mn cluster when Ca^{2+} is absent [36]. Several lines of experimental evidence support this proposal [36–38]. The results of the present paper have relevance to our understanding of the proposed chelator binding effect.

The occupancy of the Ca^{2+} site by Mn^{2+} does not reverse the effects of Ca^{2+} depletion nor of the binding of chelators to PSII. This result may be taken then as indicating that the Ca^{2+} site and the chelator binding site do not overlap. This is consistent with the suggestions that, (1) the chelator may bind directly to the Mn cluster [36–38] and (2) the Ca^{2+} and the Mn cluster do not share the same co-ordination sphere (the present work and see Ref. [22]). The fact that the displacement of a single Mn^{2+} by Ca^{2+} occurs simultaneously with (a) the loss of the stable S_2 multiline, (b) the loss the ability to observe the S_3 signal and, in consequence, (c) the restoration of activity, indicates that Ca^{2+} binding affects the environment of the Mn cluster resulting in debinding of the chelator. The lack of such an effect when Mn occupies the site could indicate a specific conformation adopted by the protein when Ca^{2+} occupies the site but which does not occur when other ions (with the exception of Sr^{2+}) occupy the site.

Once again however, by invoking a conformation change induced specifically by Ca^{2+} binding, the possibility is raised that the Ca^{2+} itself could be shifted closer to the Mn and it could thus be directly responsible for the proposed displacement of the chelator. (A conformational change of the 33 kDa polypeptide measured by FTIR has been reported to occur upon Ca^{2+} binding and seems to affect oxygen evolution under certain conditions [41]. It is not clear how this relates to the calcium effect reported here, however, it has recently been concluded that the Ca^{2+} site responsible for the EPR effects which are a focus of the present study is unaffected by removal of the 33 kDa polypeptide [12,42].)

The relaxation of $TyrD^{\cdot}$ appears to be markedly enhanced when Mn^{2+} occupies the Ca^{2+} site as detected when the Mn cluster is in the S_1 state. The effect is smaller than that seen from the Mn cluster itself when in the paramagnetic S_2 state. In future work, it may be possible to use this dipolar coupling to deduce the possible locations of the Ca^{2+} relative to the Tyr radical, using saturation recovery methods and high field EPR.

The present work indicates that Mn^{2+} can be specifically bound in the Ca-site and that, by using the method described here, this can be done in the majority of the centres. The lack of obvious spectroscopic effects indicate that this Mn^{2+}-occupied Ca^{2+}-site is outside the co-ordination sphere of the Mn cluster. Future spectroscopic studies of the Mn-substituted system reported here may provide further information on the whereabouts and nature of the site.

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